Remarks

Entry of this amendment is respectfully requested. Claims 1, 12 and 18-20 are pending in the instant application. Claims 1, 12, and 18 to 20, stand rejected. Claims 1, 12, 18, and 20 are amended herein. Claim 20 is objected to. There is no issue of new matter.

The amended claims are directed to mutant allergens of pro-DerP1 from *Dermatophagoides pteronyssinus*. Pro-DerP1 is an enzymatically inactive form of DerP1. None of the prior art documents disclose mutant versions of the *D. pteronyssinus* pro-DerP1 protein as taught by the present invention. Further, none of the documents suggest introducing mutations into the *D. pteronyssinus* pro-DerP1 protein in order to obtain an enzymatically inactive protein which has reduced allergenicity compared to wild type DerP1 protein, and which may be used to treat an individual suffering from an allergy to the DerP1 protein.

Claim Objections

Claim 20 stands objected to because of the following of improper Markush format. Without conceding the validity of this rejection, and to further prosecution, the Applicants herein amend Claim 20 in a manner that obviates the asserted basis for this rejection. The Applicants respectfully assert that, due to the amendment, this rejection is now moot.

Claim Rejections Under 35 USC § 112

Claim 1 stands rejected under 35 U.S.C. §112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. Without conceding the validity of this rejection, Applicants have elected to present the invention in different terms, which terms obviate the asserted basis for this rejection. Applicants respectfully assert that due to the amendments made to the existing claims, this rejection is now moot. Specifically, Applicants have amended claim 1, to include a reference sequence to show where the mutation is different from the reference sequence as suggested by the Examiner and more particularly point out and distinctly claim that which Applicants regard as the subject matter of their invention. The amendment is fully supported by the specification.

Specifically, support for the amendments to Claim1, SEQ ID NO. 1, referring to one possible mutant, within the scope of the invention, is identified in SEQ ID NO. 1, in Figure 8.

Claims 1, 12, and 18 to 20 stand rejected under 35 U.S.C. §112, first paragraph, as allegedly:

> …not reasonably providing enablement for: **a recombinant mutant pro-DerP1 allergen from Dermatophagoides pteronyssinus wherein said mutant allergen** comprises **an alanine substitution of Cys132 residue of pro-DerP1**; and a vaccine comprising **a recombinant mutant pro-DerP1 allergen** and an adjuvant. (emphasis provided by Examiner)

In addition, the Examiner states that:

> Claims 1 and 18-20, recite "a recombinant mutant pro-DerP1 allergen" without reference to a specific allergen sequence. This term encompasses a very large number of allergen mutants, including mutants of as yet discovered pro-DerP1 variants, that have not been disclosed in the specification. As stated supra, the limitation of a mutation at Cys132 does not further limit the claim in the absence of a reference sequence.

Without conceding the validity of this rejection, Applicants have elected to present the invention in different terms, which terms obviate the asserted basis for this rejection. Applicants respectfully assert that due to the amendments made to the existing claims, this rejection is now traversed. Specifically, Applicants have amended claim 1 and 12, to include a reference sequence to show where the mutation is different from the reference sequence as suggested by the Examiner to more particularly point out and distinctly claim that which Applicants regard as the subject matter of their invention. The amendments are fully supported by the specification.

Specifically, support for the amendments to Claim1 and 12, SEQ ID NO. 1, referring to one possible mutant, is identified in SEQ ID NO. 1 in Figure 8.

In addition, in the context of the specification, the meaning of the term "a recombinant mutant pro-DerP1 allergen" would be clear to a person skilled in the art. Specifically, it is clear from the application as filed and previously published references that the term "DerP1" is used to refer to a proteolytically active, mature protein having 222 amino acid residues (see, for example, page 2 line 31 to page 3 line 2 and SEQ ID NO. 1 (DerP1) amino acids 99 to 320). The mature protein may additionally have attached to it a pro-peptide section resulting in an enzymatically inactive pro-form of DerP1 which is referred to by the term "pro-DerP1" (see SEQ ID NO. 1 amino acids (pro) 19 to 98 and amino acids (pro-DerP1) 19 to 320, Examples, and page 37 of post publication reference by some of the inventors: Massaer *et al.*, High-Level Expression in Mammalian Cells of Recombinant House Dust Mite Allergen ProDer p 1 with Optimized Codon Usage, Int Arch Allergy Immunol

2001;125:32-43). Further, pro-DerP1 may have attached to it a leader or signal peptide which is referred to as a pre-peptide section and results in a "pre-pro-protein" (see, for example, page 7 line 8 and the Examples and SEQ ID NO. 1 amino acids (pre) 1 to 18 and amino acids (pre-pro-DerP1) 1 to 320). Of course, in the explanation above, the Cys132 residue of the DerP1 pre-pro-protein (corresponding to position Cys34 of the mature protein) in SEQ ID NO. 1 has been changed to alanine.

Thus, the meaning of the terms "DerP1", "pro-DerP1" and "pre-pro-protein" can be summarized as follows:

"DerP1"	mature protein
"pro-DerP1"	polypeptide consisting of a pro-peptide section and the mature protein
"pre-pro-protein"	polypeptide consisting of a pre-peptide section, a pro-peptide section and the mature protein

Accordingly, claim 1 and 12 refer to mere examples of a mutant of Pro-DerP1, i.e. a mutant of a polypeptide comprising a pro-peptide section and the mature protein.

The nature of the mutation is defined according to claim 1 as the substitution of a specific cysteine residue for an alanine residue. As indicated at page 7 lines 6 to 9 of the application as filed, the substituted cysteine residue corresponds to position 132 of the pre-pro-protein, which in turn corresponds to position 34 of the mature protein. Claim 1 has been amended in order to further clarify the position of the cysteine residue by referring to its position in the pre-pro-protein. A person skilled in the art would be familiar with the numbering of a particular amino acid residue by reference to its position in the pre-pro-protein and the mature protein to avoid confusion as to which residue is intended.

In addition, the Examiner alleges that the specification disclosure does not enable one skilled in the art to practice the invention without an undue amount of experimentation. Applicants submit that there is a reasonable correlation between the scope of the claims and scope of the enablement set forth. For example, the application states, at page 4, lines 4-13:

> The allergens of the present invention are recombinantly produced. Der p1 proteolytic activity can be impaired by introducing mutations into the cDNA or genomic DNA, either at the enzymatically active site, or at the site of cleavage between the propeptide and the mature molecule. Said mutant allergen having the following advantages over the wild-type allergen: 1) increases the Th1-type aspect of the immune responses in comparison to those stimulated by the wild type allergen, thereby leading to the suppression of allergic potential of the vaccinated host, and 2) having reduced allergenicity thus being more suitable for systemic administration of high doses of the immunogen, 3) will induce

DerP1 specific IgG which compete with IgE for the binding of native DerP1.

and at page 6, line 30, to page 7, line 5:

The invention is not limited to the specifically disclosed sequence, but includes any proteolytic allergen which has been mutated to remove some or all of its proteolytic activity, whilst retaining the ability to stimulate an immune response against the wild-type allergen. The proteolytic activity of the mutant allergens may be compared to the wild type by a CD23 cleavage assay according to Shultz *et al.*, 1995, European Journal of Immunology, 25, 3191-3194), or enzymatic degradation of substrates described in Machado et al., 1996, Eur.J.Immunol., 26, 2972-2980. The immunogenicity of the mutant allergen may be compared to that of the wild-type allergen by various immunologicals assays. The cross-reactivity of the mutant and wild-type allergens may be assayed by *in vitro* T-cell assays after vaccination with either mutant or wild-type allergens. Briefly, splenic T-cells isolated from vaccinated animals may be restimulated *in vitro* with either mutant or wild-type allergen followed by measurement of cytokine production with commercially available ELISA assays, or proliferation of allergen specific T cells may be assayed over time by incorporation of tritiated thymidine. Also the immunogenicity may be determined by ELISA assay, the details of which may be easily determined by the man skilled in the art. Briefly, two types of ELISA assay are envisaged. First, to assess the recognition of the mutant DerP1 by sera of mice immunized with the wild type Der p1; and secondly by recognition of wild type DerP1 allergen by the sera of animals immunised with the mutant allergen. Briefly, each wells will be coated with 100 ng of purified wild type or mutated Der p1 overnight at 4°C. After incubating with a blocking solution (TBS-Tween 0.1% with 1% BSA) successive dilutions of sera will be incubated at 37°C for 1 hour. The wells are washed 5 times, and total IgG revealed by incubating with an anti-IgG antibody conjugated with Alkaline phosphatase.

The reduction of enzymaticaly active allergen or DerP1 may be performed by introducing mutations into the native sequence before recombinantly producing the inactivated mutants. This may be achieved by: introducing substitutions, deletions, or additions into the active sites; by inserting, deleting, or substituting residues in regions of processing the inactive pro-enzyme into the active mature protein; or by altering the three dimensional structure of the protein such that enzymatic activity is lost, this may be achieved, amongst others, by expressing the protein in fragments, or by deleting cysteine residues involved in disulphide bridge formation, or by deleting or adding residues such that the tertiary structure of the protein is substantially altered.

In consideration of the amended claims and specification disclosure, the Applicants assert that the skilled artisan can envision the pro-DerP1 allergens recited in the instant claims. The key to analyzing an "undue experimentation attack" on the enablement of a patent, and therefore of an anticipatory reference, is in determining what is "undue," because some trial and error is permissible. *See W.L. Gore & Assoc. v. Garlock, Inc.*, 721 F.2d 1540, 1557, 220 U.S.P.Q. 303, 316 (Fed. Cir. 1983) ("Assuming some experimentation were needed, a patent is not invalid because of a need for experimentation. A patent is invalid only when those skilled in the art are required to engage in *undue* experimentation to practice the invention." (cite omitted) (emphasis in original)). *cert. denied*, 469 U.S. 851 (1984).

A skilled artisan would focus on mutations disclosed herein and experimentation with pro-DerP1 mutations that yield identifying characteristics and based on the representative description of the structural and functional properties of the claimed and disclosed invention as described above and further in the specification.

As will be recognized, the enablement requirement of § 112 is satisfied so long as a disclosure contains sufficient information that persons of ordinary skill in the art having the disclosure before them would be able to make and use the invention. *In re Wands*, 8 U.S.P.Q.2d 1400 (Fed. Cir. 1988) (the legal standard for enablement under § 112 is whether one skilled in the art would be able to practice the invention without undue experimentation). In this respect, the following statement from *In re Marzocchi*, 169 U.S.P.Q. 367, 369-370 (C.C.P.A. 1971), is noteworthy:

> The only relevant concern of the Patent Office under these circumstances should be over the truth of any such assertion. The first paragraph of § 112 requires nothing more than objective enablement. How such a teaching is set forth, either by the use of illustrative examples or by broad terminology, is of no importance.
>
> As a matter of Patent Office practice, then, a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented ***must*** be taken as in compliance with the enabling requirements of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied upon for enabling support. (emphasis added)

Any assertion by the Patent Office that an enabling disclosure is not commensurate in scope with the protection sought must be supported by evidence or reasoning substantiating the doubts so expressed.

In view of the forgoing remarks, the Applicants respectfully submit that they have overcome all grounds of the Examiner's rejection under 35 U.S.C. §112, second paragraph, and that rejection should be withdrawn.

Claim Rejections Under 35 USC § 103

Claims 1 stands rejected under 35 USC §103(a) as being allegedly unpatentable over Robinson *et al.* in view of WO 94/05790. The Examiner posits that:

> It would have been obvious for one skilled in the art at the time of the invention was made to modify the corresponding cysteine residue in the pro-DerP1 form of the allergen because Robinson *et al.* teaches the cysteine 34 (corresponding to cysteine 132 in the pro-form) is a catalytic site of the enzyme and enzymatic activity of DerP1 is related to its immunogenicity.

Applicants respectfully disagree. Robinson *et al.* describes the enzymatic activity of certain allergens and discusses the significance of the structure of such molecules in how they may be recognized by the immune system. At page 19, left hand column, last paragraph, Robinson *et al.* state (emphasis added):

> "The expression of active DerP1 and the construction of genetically engineered mutants which abolish or reduce enzymatic activity, should clarify the role of proteolytic activity in allergic reactions. For example it should be possible to replace either the active site C with S or A, or replace the active site H residue with F, Y and T with no predictable effect on the structure of the protein, and to investigate whether proteolytic activity is crucial in mediating an IgE response in cell culture and whole animal systems."

It is apparent that according to Robinson *et al.* the term "DerP1" is used to refer to the enzymatically active, mature protein. The passage at page 19 describes a number of possible mutations at the active site of DerP1. However, there is no clear and unambiguous disclosure of a recombinant mutant pro-DerP1 allergen *i.e.* a mutant of the enzymatically inactive pro-form of DerP1. More particularly, there is no teaching regarding a recombinant mutant pro-DerP1 having an alanine substitution of the Cys 132 residue of the pre-pro-protein (corresponding to position 34 of the mature protein) according to claim 1.

WO94/05790 discloses the sequence of DerP1 and an incomplete/proposed pro-DerP1 sequence in Figures 1A and 1B (see page 17, lines 34-38). The positive amino acid numbers correspond to the sequence of the mature, excreted DerP1, whereas the negative numbers refer to the proposed, transient pre- and preproenzyme forms of DerP1 (page 2 lines 30-37). WO94/05790 also describes the isolation of a full length DerP1 cDNA clone, which was used to express DerP1 in fusion with a glutathione transferase molecule (Example 2, page 18, lines 26-29).

As described above, the pro-DerP1 sequence proposed in WO94/05790 is different from the pro-DerP1 sequence in the present invention. The authors of WO94/05790, propose that the pro- sequence begins at Phe(-12) and ends at Glu(-1). The pro- sequence of the present invention begins at what is equivalent to Arg(-79) (See SEQ ID NO. 1 amino acids (pro) 19 to 98 and amino acids (pro-DerP1) 19 to 320, Examples, and page 37 of post publication reference by some of the inventors: Massaer *et al.*, High-Level Expression in Mammalian Cells of Recombinant House Dust Mite Allergen ProDer p 1 with Optimized Codon Usage, Int Arch Allergy Immunol 2001;125:32-43). It is clear from Figure 1A that WO94/05790 does not disclose amino acids -79 to -23. WO94/05790 only discloses sequence beginning at Lys(-22), See Figure 1A.

Without conceding the validity of this rejection, and to further prosecution, the Applicant herein amends the pending claims terms to obviate the asserted basis for this rejection. Specifically, the Applicant has amended Claim 1 to recite "A recombinant mutant pro-DerP1 allergen from *Dermatophagoides pteronyssinus,* wherein said mutant allergen comprises an alanine substitution of the Cys132 residue of DerP1 pre-pro-protein (SEQ ID NO. 1)." The Applicant respectfully asserts that, due to the arguments and amendments made to the existing claims herein, this rejection is now traversed.

In view of the foregoing remarks and claim amendments, the Applicant respectfully requests that the Examiner withdraw her rejection based on Claim 1 under 35 U.S.C. §103.

Claims 1 and 18-20 stand rejected under 35 USC §103(a) as being allegedly unpatentable over Robinson *et al.* in view of WO 94/05790 and US 5,762,943. The Examiner posits that "it would have been obvious for one skilled in the art to add 3D-MPL to the allergen pharmaceutical composition taught by Robinson *et al.* and WO 94/05790 because the '943, patent teaches that it would make the allergen immunotherapy safer safer.

Due to the arguments made in the preceding paragraphs, Applicants respectfully disagree. Robinson *et al.* discloses “DerP1” to refer to the enzymatically active, mature protein, not the enzymatically inactive pro-form of DerP1 (pro-DerP1). In addition, the pro-DerP1 sequence proposed in WO94/05790 is different from the pro-DerP1 sequence in the present invention. Therefore, there is no way, separately or in combination, that one skilled in the art would or could combine the above references to arrive at the present invention.

Without conceding the validity of this rejection, and to further prosecution, the Applicant herein amends the pending claims terms to obviate the asserted basis for this rejection. Specifically, the Applicant has amended Claim 1 to recite “A recombinant mutant pro-DerP1 allergen from *Dermatophagoides pteronyssinus,* wherein said mutant allergen comprises an alanine substitution of the Cys132 residue of DerP1 pre-pro-protein (SEQ ID NO. 1).” The Applicant respectfully asserts that, due to the arguments and amendments made to the existing claims herein, this rejection is now traversed.

In view of the foregoing remarks and claim amendments, the Applicant respectfully requests that the Examiner withdraw her rejection based on Claims 1 and 18-20 under 35 U.S.C. §103.

The Applicants reserve the right to prosecute, in one or more patent applications, the claims to non-elected inventions, the claims as originally filed, and any other claims supported by the specification. The Applicants thank the Examiner for the Office Action and believe this response to be a full and complete response to such Office Action. Accordingly, favorable reconsideration and allowance of the pending and new claims is earnestly solicited. If it would expedite prosecution of this application, the Examiner is invited to confer with the Applicants’ undersigned attorney.

Respectfully submitted,

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Attachment: Massaer *et al.*, High-Level Expression in Mammalian Cells of Recombinant House Dust Mite Allergen ProDer p 1 with Optimized Codon Usage, Int Arch Allergy Immunol 2001;125:32-43

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High-Level Expression in Mammalian Cells of Recombinant House Dust Mite Allergen ProDer p 1 with Optimized Codon Usage

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Key Words

Der p 1 · ProDer p 1 · Allergen · Codon usage · Synthetic gene

Abstract

Background: The major house dust mite allergen Der p 1 is associated with allergic diseases such as asthma. Production of recombinant Der p 1 was previously attempted, but with limited success. The present study describes the expression of recombinant (rec) ProDer p 1, a recombinant precursor form of Der p 1, in CHO cells. ***Methods:*** As optimization of the codon usage may allow successful overexpression of protein in mammalian cells, a synthetic gene encoding ProDer p 1 was designed on the basis of the codon usage frequently found in highly expressed human genes. Gene synthesis was accomplished from a set of 14 mutually priming overlapping oligonucleotides and after two runs of polymerase chain reaction. ***Results:*** COS cells transiently transfected with the synthetic ProDer p 1 gene produced up to 5–10 times as much ProDer p 1 compared with the expression level obtained after transfection with the authentic gene. To stably express the recombinant allergen, CHO-K1 cells were transfected with the ProDer p 1 synthetic gene, and one amplified recombinant clone produced up to 30 mg of recProDer p 1 per liter in the culture medium before purification. recProDer p 1 was secreted as an enzymatically inactive single-chain molecule presenting three glycosylated immunoreactive forms of 41, 38 and 36 kD. When examined with respect to direct binding, recProDer p 1 and natural Der p 1 displayed very similar IgE reactivities. However, IgE inhibition and histamine release assays showed a much higher reactivity to natural Der p 1 compared to recProDer p 1. ***Conclusions:*** These data indicated that codon optimization represents an attractive strategy for high-level production of allergen in mammalian cells.

Introduction

The allergens from the house dust mite *Dermatophagoides pteronyssinus* have long been recognized to be associated with allergic hypersensitivity reactions such as asthma [1]. Amongst these molecules, Der p 1 is an immunodominant allergen which elicits the strongest IgE-mediated immune response [2, 3]. The cysteine proteinase activity of Der p 1 was shown to amplify its potent allergenicity [4, 5].

The Der p 1-encoding cDNA sequence reveals that, like many mammalian and plant proteinases, Der p 1 is synthetized as an inactive preproenzyme of 320 amino acid residues which is subsequently processed into a 222-amino acid mature form [6, 7]. The mechanism of matu-

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ration of ProDer p 1 is not known to date, but it is thought that the allergen is processed by the cleavage of the 80-residue proregion.

Mature Der p 1 has been successfully purified from whole house dust mite culture, but with a low overall yield [8]. Recombinant production of allergens represents an efficient way to obtain defined materials with high yields for a variety of experimental procedures such as immunological studies, diagnosis, treatment of IgE-mediated allergic disorders by immunotherapy and understanding structure-function relationships [9]. Previous attempts to express Der p 1 in bacteria and yeast indicated that the allergen was poorly expressed and mainly in an insoluble form [10–12]. Moreover, recombinant Der p 1 produced in bacteria was shown to have weak IgE-binding activity. The recombinant protein expressed in yeast was recognized by specific IgE at, however, a lower level than the natural protein. Recently, we reported the successful production of a recombinant secreted form of ProDer p 1 (recProDer p 1) in insect cells [13]. recProDer p 1 displayed a similar immunoreactivity towards IgE and monoclonal and polyclonal IgG antibodies compared to natural Der p 1.

The *Drosophila* expression system used in our previous study presented some disadvantages in comparison with the production of recombinant proteins in mammalian cells. Indeed, plasmid transfections and hygromycin selection of S2 insect cells led to polyclonal recombinant cell lines instead of a population of defined clones. *Drosophila* cell culture is unstable when performed in multitray chambers, and no more than three successive harvests can be performed.

The present paper reports the cloning and expression of recProDer p 1 secreted by CHO cells. As the low GC content of the ProDer p 1 gene (38%) may impair expression in mammalian cells [14, 15], a synthetic ProDer p 1 gene was generated in which the codon usage was optimized for expression in CHO cells. The results showed that optimization of the codon usage is essential for ProDer p 1 expression at a high level in CHO cells.

Materials and Methods

Construction of a ProDer p 1 Synthetic Gene

A ProDer p 1 gene was synthesized using a set of 14 partially overlapping oligonucleotides. These primers were designed based on the codon preference of highly expressed human genes, and were produced by a 394 DNA/RNA Applied Biosystem synthetizer. The degenerately encoded amino acids were not encoded by the most prevalent codons but taking the frequencies of the individual codons into account. For example, the histidine residue is encoded by CAC or CAT with a respective frequency of 79 and 21% in highly expressed human genes. Consequently, we attempted to follow the same codon frequency instead of selecting only the CAC codon for each histidine residue in the synthetic ProDer p 1. The native Der p 1 signal sequence was exchanged with the highly efficient leader peptide of the VZV glycoprotein E (gE) to facilitate secretion. The oligonucleotides were as follows:

5′GAAGCTTCGGGCGAATTGCGTGGTTTTAAGTGACTATA-
TTCGAGGGTCGCCTGTAATATGGGGACAGTTAATAAA-
CCTGTGGTGGGGGTATTGATGGGGTTCGGAATTATC-
ACG3′ (oligo 1, coding);

5′GAAGGCTTTCTTGTATTCCTCGAAGGTCTTAATGGAGC-
TCGGCCGTGCTCTGACCGGATTCGTTATACGCAAGGT-
ACCCGTGATAATTCCGAACCC3′ (oligo 2, noncoding);

5′GGAATACAAGAAAGCCTTCAACAAGAGCTATGCCACCT-
TCGAGGACGAGGAGGCCGCGCGCAAGAACTTCCTGG-
AAAGCGTGAAATACGTGCAGAGC3′ (oligo 3, coding);

5′GTCTTAAGGTGTTCGAAAGCCTCGGCGCTCATCAGGAA-
CCGGTTCTTGAACTCGTCTAAAGACAGGTCGGACAGG-
TGATTTATAGCCCCGCCGTTGCTCTGCACGTATTTC-
AC3′ (oligo 4, noncoding);

5′CTTTCGAACACCTTAAGACCCAGTTTGATCTCAACGCG-
GAGACCAACGCCTGCAGTATCAACGGCAATGCCCCCG-
CTGAGATTGATCTGCGCC3′ (oligo 5, coding);

5′GACTCTGTCGCGGCCACGCCTGAAAAGGCCCAACAAGA-
CCCGCAGCCGCCTTGCATGCGGATGGGAGTCACGGT-
CCTCATCTGGCGCAGATCAATCTCAG3′ (oligo 6, noncoding);

5′GTGGCCGCGACAGAGTCGGCATACCTCGCGTATCGGA-
ATCAGAGCCTGGACCTCGCTGAGCAGGAGCTCGTTG-
ACTGCGCCTCCCAACACGG3′ (oligo 7, coding);

5′GCTACGTATCGGTAATAGCTTTCCTGCACGACGCCATT-
ATGCTGGATGTATTCGATACCTCTGGGAATCGTATCC-
CCATGACATCCGTGTTGGGAGGCGC3′ (oligo 8, noncoding);

5′GCTATTACCGATACGTAGCTAGGGAGCAGTCCTGCCGC-
CGTCCTAACGCACAGCGCTTCGGCATTTCCAATTATT-
GCCAGATCTACC3′ (oligo 9, coding);

5′CCTTGATTCCGATGATGACAGCGATGGCGCTGTGCGTC-
TGCGCCAGGGCCTCCCTGATCTTGTTGGCATTAGGG-
GGGTAGATCTGGCAATAATTG3′ (oligo 10, noncoding);

5′GTCATCATCGGAATCAAGGATCTGGACGCATTCCGGCA-
CTATGACGGGCGCACAATCATCCAGCGCGACAACGGA-
TATCAGCCAAACTACC3′ (oligo 11, coding);

5′GTAGTCCACCCCCTGGGCGTTCGAGTAACCCACGATGT-
TGACCGCGTGGTAGTTTGGCTGATATCC3′ (oligo 12, noncoding);

5′CCAGGGGGTGGACTACTGGATCGTGAGAAACAGTTGG-
GACACTAACTGGGGCGACAACGGCTACGGCTACTTCG-
CCGCCAAC3′ (oligo 13, coding);

5′GCTCTAGACTCGAGGGATCCTTACAGGATCACCACGTA-
CGGGTACTCCTCGATCATCATCAGGTCGATGTTGGCG-
GCGAAGTAGC3′ (oligo 14, noncoding).

The oligonucleotides were incubated together for the amplification of a synthetic ProDer p 1 gene in a polymerase chain reaction (PCR). Typically, PCR was conducted using High Fidelity Polymerase (Boehringer) with the following conditions: 30 cycles, denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and elongation at 72 °C for 30 s. The generated products were amplified using the 3′

and 5′ terminal primers (oligo 1 and 14) in the same conditions. The resulting 1,080-bp fragment was cloned into a pCRII-TOPO cloning vector (Invitrogen). The resulting plasmid, pNIV4845, was used to transform the *Escherichia coli* strain TOP 10 (Invitrogen).

Construction of a Humanized ProDer p 1 Expression Vector

As the sequencing of eight bacterial clones demonstrated some mutations in the synthetic ProDer p 1 gene, the plasmid for stable expression was generated starting from four ProDer p 1 DNA fragments derived from bacterial clones carrying pNIV4845. Clones 5 and 20 were submitted to double digestions by *Hin*dIII-*Bss*HII and *Sph*I-*Bgl*II, respectively, to isolate the 228-bp *Hin*dIII-*Bss*HII and 272-bp *Sph*I-*Bgl*II ProDer p 1 DNA fragments. Clone 7 was restricted with *Bss*HII-*Sph*I and *Bgl*II-*Xba*I to generate the 239-bp *Bss*HII-*Sph*I and 329-bp *Bgl*II-*Xba*I ProDer p 1 DNA fragments. These fragments were inserted into the *Hin*dIII-*Xba*I-cut pEE14 expression vector (Celltech) [16] to give the final plasmid pNIV4846. The correct recombinants were confirmed by DNA sequencing.

Transient Transfections and Selection of recProDer p 1-Producing Stable CHO-K1 Lines

To determine the expression levels of recProDer p 1, COS cells (ATCC) were transiently transfected with 10 µg of pNIV4846 or pNIV4853, the latter plasmid carrying the authentic ProDer p 1 gene, by calcium phosphate coprecipitation. For stable recProDer p 1 expression, CHO-K1 cells (ATCC) were transfected with pNIV4846 plasmid by lipofection. After a 3-week 25 µ*M* methionylsulphoximin (MSX; Sigma) selection, one round of gene amplification was carried out with 100 µ*M* MSX.

Expression of the Recombinant Allergen in CHO Cells

The best-producing recombinant CHO-K1 clone was cultured in cell factories in GMEM medium (Invitrogen) supplemented with 2% fetal calf serum (Gibco). Spent culture medium was harvested every 72 h and stored at –20 °C until purification.

Purification of Natural Der p 1 From Natural Mite Whole Body Extracts

Purification of natural Der p 1 from whole mite culture was performed as previously described [13]. Briefly, *D. pteronyssinus* extracts were submitted to $(NH_4)_2SO_4$ precipitation to 60% saturation. The precipitate, collected by ultracentrifugation and resuspended in PBS containing 1 *M* $(NH_4)_2SO_4$, was applied to a Resource Phenyl column (Pharmacia) equilibrated in PBS containing 1 *M* $(NH_4)_2SO_4$. Der p 1 was eluted from the column with water. After pH and conductivity adjustments of the Der p 1-enriched fractions, the pool was applied to a Q sepharose fast flow column (Pharmacia) equilibrated in 20 m*M* Tris-HCl, pH 9. Der p 1 was eluted by the addition of 200 m*M* NaCl to the starting buffer. Der p 1 purification was achieved by gel filtration chromatography onto a superdex-75 column (Pharmacia) equilibrated in PBS, pH 7.3. Purified Der p 1 was concentrated and stored at –20 °C.

Purification of recProDer p 1 From CHO Spent Culture Medium

CHO spent culture medium was diluted two times with water and the pH was adjusted to 7.2. The modified supernatant was loaded to a Q sepharose fast flow column (5 × 10 cm; Pharmacia) equilibrated in 20 m*M* Tris-HCl, pH 7.2, which was coupled to a hydroxyapatite column (2.6 × 15 cm; Bio-Rad) conditioned in the same buffer. The flow-through containing recProDer p 1 of both columns was adjusted to pH 9 and applied to a Q sepharose fast flow column (1.6 × 10 cm) equilibrated in 20 m*M* Tris-HCl, pH 9. The column was washed with the starting buffer and with the same buffer supplemented with 100 m*M* NaCl. ProDer p 1 was eluted by a linear NaCl gradient (100–300 m*M*, 15 column volumes). The recProDer p 1-enriched fractions were pooled and concentrated by ultrafiltration onto a Filtron membrane (Omega series, cutoff: 10 kD). recProDer p 1 purification was achieved by gel filtration chromatography onto a superdex-75 column (1 × 30 cm; Pharmacia) equilibrated in PBS, pH 7.3. Purified recProDer p 1 was concentrated and stored at –20 °C.

SDS-PAGE and Western Blot Analysis

Proteins were analyzed by SDS-PAGE on 12.5% polyacrylamide gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes using a semidry transblot system (Bio-Rad). Membranes were saturated for 30 min with 0.5% Instagel (PB Gelatins) in 50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl and 0.1% Tween 80 (TBS-T) and incubated with rabbit polyclonal serum raised against ProDer p 1 peptide 245–267 diluted in blocking solution (1/5,000) (kindly provided by Dr. Pestel, Institut Pasteur de Lille, France) [17]. Immunoreactive materials were detected using alkaline phosphatase-conjugated goat anti-rabbit antibodies (Promega; 1/7,500) and 5-bromo-4-chloro-3-indolylphosphate (Boehringer)/nitroblue tetrazolium (Sigma) as substrates.

Glycan Analysis

Carbohydrate analysis was carried out with the Glycan Differentiation Kit (Boehringer) using the following lectins: *Galanthus nivalis* agglutinin, *Sambucus nigra* agglutinin, *Maackia amurensis* agglutinin, peanut agglutinin, and *Datura stramonium* agglutinin. Briefly, purified proteins were transferred from SDS-PAGE onto nitrocellulose membranes. The membranes were incubated with the different lectins conjugated to digoxigenin. Complexes were detected with antidigoxigenin antibodies conjugated to alkaline phosphatase.

Enzymatic Assays

Enzymatic assays were performed in 50 m*M* Tris-HCl, pH 7, containing 1 m*M* EDTA and 20 m*M* *L*-cysteine at 25 °C in a total volume of 1 ml. Hydrolysis of Cbz-Phe-Arg-7-amino-4-methylcoumarin (Cbz-Phe-Arg-AMC) and Boc-Gln-Ala-Arg-7-amino-4-methylcoumarin (Boc-Gln-Ala-AMC) (Sigma) (both substrates at a final concentration of 100 µ*M*) was monitored using an SLM 8000 spectrofluorimeter with λ_{ex} = 380 nm and λ_{em} = 460 nm. Assays were started by the addition of cysteine-activated allergen to a final concentration of 100 n*M*. Before any assay, purified Der p 1 or recProDer p 1 was incubated with a mixture of aprotinin- and *p*-aminobenzamidine-agarose resins (Sigma) to remove any putative trace of serine protease activity.

Protein Determination

The total protein concentration was determined by the bicinchoninic acid procedure (MicroBCA, Pierce) with bovine serum albumin as standard.

Der p 1 ELISA

Der p 1 or recProDer p 1 was detected with an ELISA kit using the Der p 1-specific monoclonal antibodies 5H8 and 4C1 (Indoor Biotechnologies). The Der p 1 standard (UVA 93/03) used in the assay was used at a concentration of 2.5 µg/ml.

Amino acid	Codon		High	ProDer p 1	Synthetic
Ala	GC	G	17	0	20
		A	13	21	11
		T	17	**53**	20
		C	**53**	26	**49**
Arg	AG	G	18	0	20
		A	10	0	11
	CG	G	21	0	22
		A	6	**50**	6
		T	7	44	6
		C	**37**	6	**39**
Asn	AA	T	22	48	26
		C	**78**	**52**	**74**
Asp	GA	T	25	**94**	25
		C	**75**	6	**75**
Cys	TG	T	32	**57**	29
		C	**68**	43	**71**
Gln	CA	G	**88**	0	**88**
		A	12	**100**	12
Glu	GA	G	**75**	0	**75**
		A	25	**100**	25
Gly	GG	G	24	0	25
		A	14	15	15
		T	12	**70**	10
		C	**50**	15	**50**
His	CA	T	21	**50**	25
		C	**79**	**50**	**75**
Ile	AT	A	5	0	4
		T	18	**54**	17
		C	**77**	46	**79**
Leu	TT	G	6	**75**	5
		A	2	10	5
	CT	G	**58**	0	**60**
		A	3	0	0
		T	5	5	5
		C	26	10	25
Lys	AA	G	**82**	0	**82**
		A	18	**100**	18
Phe	TT	T	20	42	17
		C	**80**	**58**	**83**
Pro	CC	G	17	0	22
		A	16	**89**	11
		T	19	0	22
		C	**48**	11	**44**
Ser	AG	T	10	23	9
		C	**34**	14	**41**
	TC	G	9	9	9
		A	5	**41**	5
		T	13	5	9
		C	28	9	27
Thr	AC	G	15	0	18
		A	14	9	18
		T	14	**55**	18
		C	**57**	36	**45**
Tyr	TA	T	36	**57**	29
		C	**74**	43	**71**
Val	GT	G	**64**	0	**65**
		A	5	17	6
		T	7	39	6
		C	25	**44**	24

Fig. 1. Codon usage of ProDer p 1 and highly expressed human (High) genes. Codon usage of a synthetic ProDer p 1 gene (synthetic) after optimization of codon usage is also represented. Percentage frequencies of individual codons are shown for each corresponding amino acid. The most prevalent codon is shown in bold.

IgE-Binding Activity

Immunoplates were coated overnight with Der p 1 or recProDer p 1 (500 ng/well) at 4 °C. Plates were then washed 5 times with 100 µl per well of TBS-T buffer and saturated for 1 h at 37 °C with 150 µl of the same buffer supplemented with 1% bovine serum albumin (Sigma). Sera from patients allergic to *D. pteronyssinus* diluted 1/8 were then incubated for 1 h at 37 °C. Out of the 95 sera used in the experiments, 16 sera ranged in their specific anti-*D. pteronyssinus* IgE values (RAST assays) from 58.1 to 99 kU/l and 79 were above the upper cutoff value of 100 kU/l. The plates were washed five times with TBS-T buffer and the allergen-IgE complexes were detected after incubation with a mouse anti-human IgE antibody (Southern Biotechnology Associates) and a goat anti-mouse IgG antibody coupled to alkaline phosphatase (dilution 1/7,500 in TBS-T buffer; Promega). The enzymatic activity was measured using the *p*-nitrophenylphosphate substrate (Sigma) dissolved in diethanolamine buffer (pH 9.8). $OD_{410\,nm}$ was measured in a Biorad Novapath ELISA reader.

For IgE inhibition assays, plates were coated with Der p 1 or recProDer p 1 at the same concentration (0.12 µ*M*). A pool of 20 human sera from allergic patients (RAST value >100 kU/l) was preincubated overnight at 4 °C with various concentrations (3.6–0.002 µ*M*) of Der p 1 or recProDer p 1 as inhibitors and added to ELISA plates. IgE binding was detected as described above.

Histamine Release

The histamine release was assayed using leukocytes from the peripheral heparinized blood of an allergic donor and by the Histamine-ELISA kit (Immunotech). Basophils were incubated with serial dilutions of recProDer p 1 or Der p 1 for 30 min at 37 °C. The total amount of histamine in basophils was quantified after cell disruption with the detergent IGEPAL CA-630 (Sigma).

Results

Synthesis of a Humanized ProDer p 1 Gene

The codon prevalence of the ProDer p 1 gene displayed many divergences compared with that used in highly expressed human genes (fig. 1). In consequence, oligonucleotides were designed for the construction of a synthetic ProDer p 1 gene to optimize the allergen expression in mammalian cells. As shown in figure 1, the final codon frequency in the synthetic ProDer p 1 gene was very similar to that used in highly expressed mammalian genes.

Synthetic ProDer p 1 was assembled from mutually priming oligonucleotides that were subsequently amplified by PCR (fig. 2). After one round of PCR, amplified products displayed a molecular weight ranging from 3,000 to 300 bp (fig. 2). A subsequent amplification with primers complementary to the 5′ end of the VZV gE leader peptide and to the 3′ end of the synthetic ProDer p 1 gene led to a 1,072-bp fragment of expected size. The amplified fragment was cloned into the pCRII cloning vector. Sequence analysis of recombinant clones revealed the presence of point mutations and deletions in the synthetic ProDer p 1 gene. Finally, the correct coding cassette was obtained after ligation of four different fragments isolated from three independent bacterial clones and in-

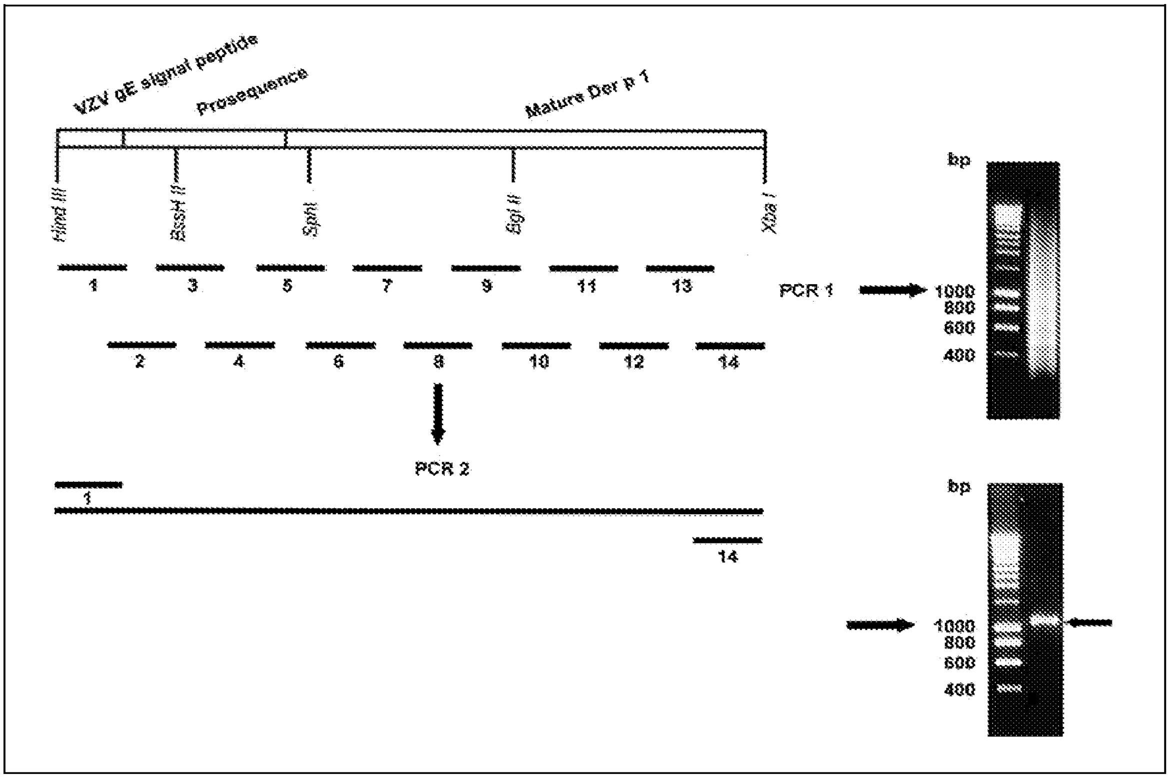

Fig. 2. PCR synthesis of ProDer p 1 cDNA. A set of 14 mutually priming oligonucleotides was used for PCR amplification of a synthetic ProDer p 1 cDNA. After one round of amplification, amplified products were submitted to a second PCR amplification using external primers (primers 1 and 14). Oligonucleotides which served as PCR templates for the synthesis are represented by solid bars. Unique restriction sites in the synthetic ProDer p 1 cDNA which were used for cloning into the eukaryotic pEE14 expression vector are shown above. Electrophoresis on agarose gel of the amplified fragments after each of the two rounds of PCR amplification is also shown.

serted in the mammalian expression vector pEE14 to give the final plasmid pNIV4846.

Figure 3 shows the sequence alignment of natural and synthetic ProDer p 1 genes.

Transient and Stable Expression of recProDer p 1

To compare the expression efficiency of the synthetic ProDer p 1 construct with the original sequence, COS cells were transfected with pNIV4846 and pNIV4853, the latter pEE14-derived plasmid carrying authentic ProDer p 1 cDNA. The recProDer p 1 expression level of the supernatants was estimated by an ELISA assay, using two anti-Der p 1 monoclonal antibodies. As shown in figure 4, the expression vector carrying the synthetic cDNA directed the recProDer p 1 synthesis more efficiently than the same vector containing the authentic gene. The expression level of the humanized ProDer p 1 gene was enhanced up to 450 ng/ml/72 h, which represents a 6-fold increase compared with the reference construction (75 ng/ml/72 h). As expected, no recProDer p 1 expression was detected using COS cells transfected by a control vector without any insert.

CHO-K1 cells were transfected with pNIV4846 and clones resistant to 25 μ*M* MSX were selected. The recProDer p 1 level assayed by ELISA indicated that three independent clones secreted recProDer p 1 at up to 11 μg/ml/72 h. Addition of sodium butyrate, a molecule previously reported to enhance the expression level of recombinant proteins in culture medium [18], did not influence the recProDer p 1 synthesis. A further amplification of 25 μ*M* MSX-resistant clones up to 100 μ*M* MSX increased recProDer p 1 expression from 26 to 34 μg/ml/72 h in culture medium. Clone 1 was used for recProDer p 1 large-scale production in cell factories and purifica-

```
                  Pro
Natural       1  CGTCCATCATCGATCAAAACTTTTGAAGAATACAAAAAAGCCTTCAACAA
Synthetic     1  CGGCCGAGCTCCATTAAGACCTTCGAGGAATACAAGAAAGCCTTCAACAA
                 R  P  S  S  I  K  T  F  E  E  Y  K  K  A  F  N  K

Natural      51  AAGTTATGCTACCTTCGAAGATGAAGAAGCTGCCCGTAAAAACTTTTTGG
Synthetic    51  GAGCTATGCCACCTTCGAGGACGAGGAGGCCGCGCGCAAGAACTTCCTGG
                  S  Y  A  T  F  E  D  E  E  A  A  R  K  N  F  L  E

Natural     101  AATCAGTAAAATATGTTCAATCAAATGGAGGTGCCATCAACCATTTGTCC
Synthetic   101  AAAGCGTGAAATACGTGCAGAGCAACGGCGGGGCTATAAATCACCTGTCC
                   S  V  K  Y  V  Q  S  N  G  G  A  I  N  H  L  S

Natural     151  GATTTGTCGTTGGATGAATTCAAAAACCGATTTTTGATGAGTGCAGAAGC
Synthetic   151  GACCTGTCTTTAGACGAGTTCAAGAACCGGTTCCTGATGAGCGCCGAGGC
                 D  L  S  L  D  E  F  K  N  R  F  L  M  S  A  E  A
                                                              Mature
Natural     201  TTTTGAACACCTCAAAACTCAATTCGATTTGAATGCTGAAACTAACGCCT
Synthetic   201  TTTCGAACACCTTAAGACCCAGTTTGATCTCAACGCGGAGACCAACGCCT
                  F  E  H  L  K  T  Q  F  D  L  N  A  E  T  N  A  C

Natural     251  GCAGTATCAATGGAAATGCTCCAGCTGAAATCGATTTGCGACAAATGCGA
Synthetic   251  GCAGTATCAACGGCAATGCCCCCGCTGAGATTGATCTGCGCCAGATGAGG
                   S  I  N  G  N  A  P  A  E  I  D  L  R  Q  M  R

Natural     301  ACTGTCACTCCCATTCGTATGCAAGGAGGCTGTGGTTCATGTTGGGCTTT
Synthetic   301  ACCGTGACTCCCATCCGCATGCAAGGCGGCTGCGGGTCTTGTTGGGCCTT
                 T  V  T  P  I  R  M  Q  G  G  C  G  S  C  W  A  F

Natural     351  CTCTGGTGTTGCCGCAACTGAATCAGCTTATTTGGCTTACCGTAATCAAT
Synthetic   351  TTCAGGCGTGGCCGCGACAGAGTCGGCATACCTCGCGTATCGGAATCAGA
                  S  G  V  A  A  T  E  S  A  Y  L  A  Y  R  N  Q  S

Natural     401  CATTGGATCTTGCTGAACAAGAATTAGTCGATTGTGCTTCCCAACACGGT
Synthetic   401  GCCTGGACCTCGCTGAGCAGGAGCTCGTTGACTGCGCCTCCCAACACGGA
                   L  D  L  A  E  Q  E  L  V  D  C  A  S  Q  H  G

Natural     451  TGTCATGGTGATACCATTCCACGTGGTATTGAATACATCCAACATAATGG
Synthetic   451  TGTCATGGGGATACGATTCCCAGAGGTATCGAATACATCCAGCATAATGG
                 C  H  G  D  T  I  P  R  G  I  E  Y  I  Q  H  N  G

Natural     501  TGTCGTCCAAGAAAGCTACTATCGATACGTTGCACGAGAACAATCATGCC
Synthetic   501  CGTCGTGCAGGAAAGCTATTACCGATACGTAGCTAGGGAGCAGTCCTGCC
                  V  V  Q  E  S  Y  Y  R  Y  V  A  R  E  Q  S  C  R

Natural     551  GACGACCAAATGCACAACGTTTCGGTATCTCAAACTATTGCCAAATTTAC
Synthetic   551  GCCGTCCTAACGCACAGCGCTTCGGCATTTCCAATTATTGCCAGATCTAC
                   R  P  N  A  Q  R  F  G  I  S  N  Y  C  Q  I  Y

Natural     601  CCACCAAATGTAAACAAAATTCGTGAAGCTTTGGCTCAAACCCACAGCGC
Synthetic   601  CCCCCTAATGCCAACAAGATCAGGGAGGCCCTGGCGCAGACGCACAGCGC
                 P  P  N  A  N  K  I  R  E  A  L  A  Q  T  H  S  A

Natural     651  TATTGCCGTCATTATTGGCATCAAAGATTTAGACGCATTCCGTCATTATG
Synthetic   651  CATCGCTGTCATCATCGGAATCAAGGATCTGGACGCATTCCGGCACTATG
                  I  A  V  I  I  G  I  K  D  L  D  A  F  R  H  Y  D

Natural     701  ATGGCCGAACAATCATTCAACGCGATAATGGTTACCAACCAAACTATCAC
Synthetic   701  ACGGGCGCACAATCATCCAGCGCGACAACGGATATCAGCCAAACTACCAC
                   G  R  T  I  I  Q  R  D  N  G  Y  Q  P  N  Y  H

Natural     751  GCTGTCAACATTGTTGGTTACAGTAACGCACAAGGTGTCGATTATTGGAT
Synthetic   751  GCGGTCAACATCGTGGGTTACTCGAACGCCCAGGGGGTGGACTACTGGAT
                 A  V  N  I  V  G  Y  S  N  A  Q  G  V  D  Y  W  I

Natural     801  CGTACGAAACAGTTGGGATACCAATTGGGGTGATAATGGTTACGGTTATT
Synthetic   801  CGTGAGAAACAGTTGGGACACTAACTGGGGCGACAACGGCTACGGCTACT
                  V  R  N  S  W  D  T  N  W  G  D  N  G  Y  G  Y  F

Natural     851  TTGCTGCCAACATCGATTTGATGATGATTGAAGAATATCCATATGTTGTCATTCTCTAA
Synthetic   851  TCGCCGCCAACATCGACCTGATGATGATCGAGGAGTACCCGTACGTGGTGATCCTGTAA
                   A  A  N  I  D  L  M  M  I  E  E  Y  P  Y  V  V  I  L  *
```

Fig. 3. Sequence comparison of wild-type (natural) and the codon-optimized (synthetic) ProDer p 1 cDNA. The deduced amino acid sequence shown below each codon is designated by the single-letter code. Starting positions of the propeptide as well as the mature Der p 1 sequence are indicated by arrows. Potential N-glycosylation sites are represented by boxes.

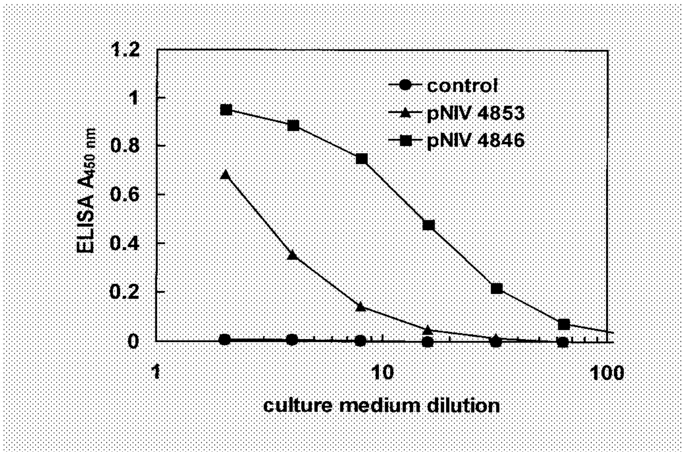

Fig. 4. Expression of synthetic and natural ProDer p 1 in transient transfection assays. Supernatants from COS cells transfected with plasmids encoding natural (pNIV4853) or synthetic ProDer p 1 (pNIV4846) were assayed for the presence of secreted recProDer p 1 in a Der p 1 ELISA. Supernatant from COS cells transfected with a plasmid without insert was used as a control.

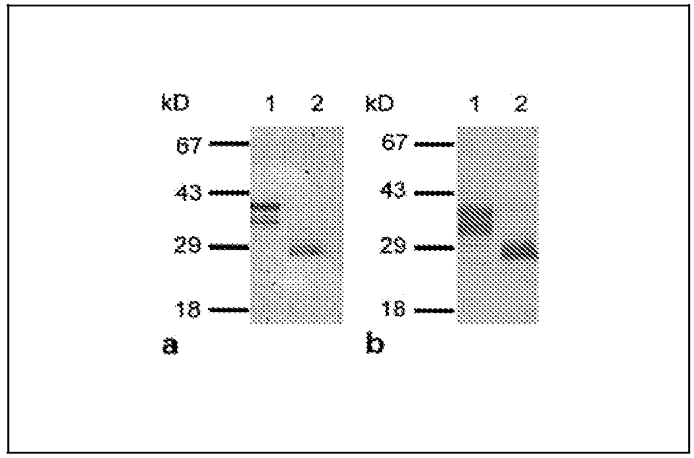

Fig. 5. Purification of recProDer p 1. Purified allergens were analyzed by SDS-PAGE and proteins were detected by Coomassie blue staining (**a**) and by immunoblotting with rabbit polyclonal serum raised against ProDer p 1 peptide 245–267 (**b**). Lane 1: purified recProDer p 1; lane 2: purified Der p 1.

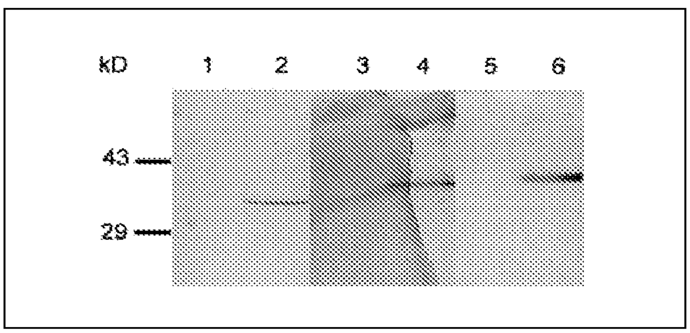

Fig. 6. Carbohydrate analysis of recProDer p 1. Glycosylation of purified allergens were analyzed by lectin staining with *G. nivalis* agglutinin (lanes 1 and 2), *D. stramonium* agglutinin (lanes 3 and 4) and *M. amurensis* agglutinin (lanes 5 and 6). Lanes 1, 3 and 5: purified Der p 1; lanes 2, 4 and 6: purified recProDer p 1.

tion. Spent culture medium was collected every 72 h and up to nine harvests were performed. In these conditions, the best recProDer p 1 expression level rose by 15 µg/ml in the culture medium before purification.

Purification of recProDer p 1

Purification of recProDer p 1 was achieved by a combination of three chromatographic steps, using anion-exchange, hydroxyapatite and gel filtration media. The final purification yield was about 6 mg of recProDer p 1 per liter of culture medium, with a recovery close to 40%. On SDS-PAGE, purified recProDer p 1 migrated as three immunoreactive species: two major bands with respective molecular weights of 41 and 36 kD and one minor band of 38 kD (fig. 5). This result indicated that the propeptide cleavage, to yield mature Der p 1, did not occur during the expression and purification steps, as natural Der p 1 migrated on SDS-PAGE as a 29-kD band. The purity of the product was greater than 90%.

Biochemical Characterization of ProDer p 1

All the recProDer p 1 species were submitted to amino-terminal amino acid sequencing. The N-terminal sequences of the 41- and 38-kD species were identical and started at residue Arg_{19}. The sequence was identified as Arg-Pro-Ser-Ser-Ile, which corresponds to the N-terminal sequence of the Der p 1 propeptide and indicates that cleavage of the VZV gE signal peptide proceeded efficiently. Surprisingly, the N-terminal sequence of the 36-kD band started at residue Ala_{38} (the obtained sequence was Ala-Thr-Phe-Glu-Asp), showing that, for the 36-kD molecule, an internal cleavage of the prosequence occurred between Tyr_{37} and Ala_{38}. Carbohydrate analysis of recProDer p 1 was performed by glycan recognition with several specific lectins (fig. 6). Among the five lectins used, only the *G. nivalis* agglutinin lectin reacted with the

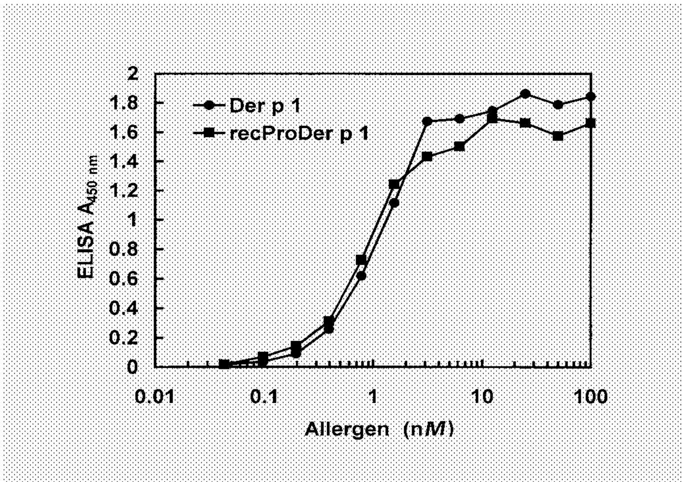

Fig. 7. Immune recognition of recProDer p 1 by monoclonal antibodies directed at Der p 1. Reactivity of Der p 1 and recProDer p 1 towards monoclonal antibodies was assayed in a two-site ELISA. Both allergens were used at the same concentration, which was determined in a total protein assay (MicroBCA, Pierce).

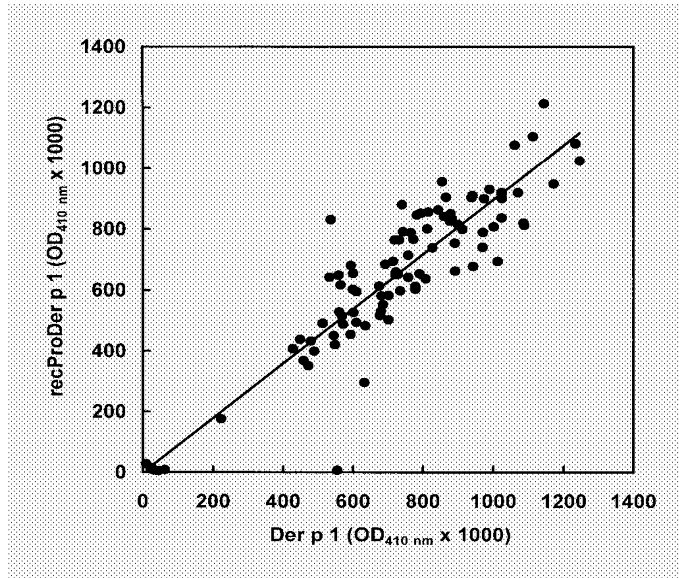

Fig. 8. Correlation between the IgE reactivity of recProDer p 1 and Der p 1. Immunoplates were coated with 500 ng/well of purified Der p 1 or recProDer p 1 and incubated with 95 sera (diluted 1/8) radioallergosorbent positive to *D. pteronyssinus*. Bound IgE was quantitated by incubation with mouse anti-human IgE and alkaline phosphatase-labeled anti-mouse IgG antibodies, followed by an enzymatic assay. Results are expressed as $OD_{410\ nm}$ values.

36-kD recProDer p 1, pointing to the presence of terminal mannose residues on this molecule, either as high-mannose N-glycan chains or as exposed mannose in hybrid chains. The 38- and 41-kD bands were recognized respectively by the *D. stramonium* agglutinin and *M. amurensis* agglutinin lectins, showing that the 38-kD molecule carried terminal galactose-linked β(1–4) to N-acetyl-glucosamine in N-glycan chains, whereas the carbohydrate structure of the upper band was terminated by sialic acid-linked α(2–3) to galactose. As previously shown [13], Der p 1 did not react with any lectin, confirming that Der p 1 is not glycosylated. The enzymatic activity of recProDer p 1 was measured using Cbz-Phe-Arg-AMC and Boc-Gln-Ala-Arg-AMC as substrates [19, 20]. recProDer p 1 was totally inactive in our assays. In the same experimental conditions, fluorogenic molecules were fully degraded within 4 min by natural Der p 1 used at the same molarity.

IgG and IgE Reactivities of recProDer p 1

recProDer p 1 was tested in ELISA assays to determine whether the recombinant allergen displayed reactivities similar to those of Der p 1 towards specific anti-Der p 1 IgG and anti-*D. pteronyssinus* IgE.

As shown in figure 7, equimolar concentrations of both allergens reacted similarly with two Der p 1-specific monoclonal and conformational antibodies, suggesting that recProDer p 1 displayed the overall structure of the natural allergen.

The IgE reactivity of recProDer p 1 and Der p 1 was compared in a direct ELISA wherein immunoplates were directly coated with Der p 1 or recProDer p 1. A set of 95 human sera with positive radioimmunosorbent tests to *D. pteronyssinus* extract was used at a dilution of 1/8. IgE titer determinations clearly showed a close correlation of IgE reactivity with both allergens, indicating that recProDer p 1 has very similar IgE-binding characteristics compared with Der p 1 ($r^2 = 0.8171$, $p < 0.0001$) (fig. 8).

The IgE reactivity of both allergens was further analyzed by competitive inhibition assays. In this case, the reactivity of IgE antibodies towards coated Der p 1 or recProDer p 1 was competed with preincubation of human sera with various concentrations of soluble Der p 1 or recProDer p 1. As shown in figure 9, the overall binding inhibition pattern after Der p 1 incubation was different to that observed after recProDer p 1 incubation.

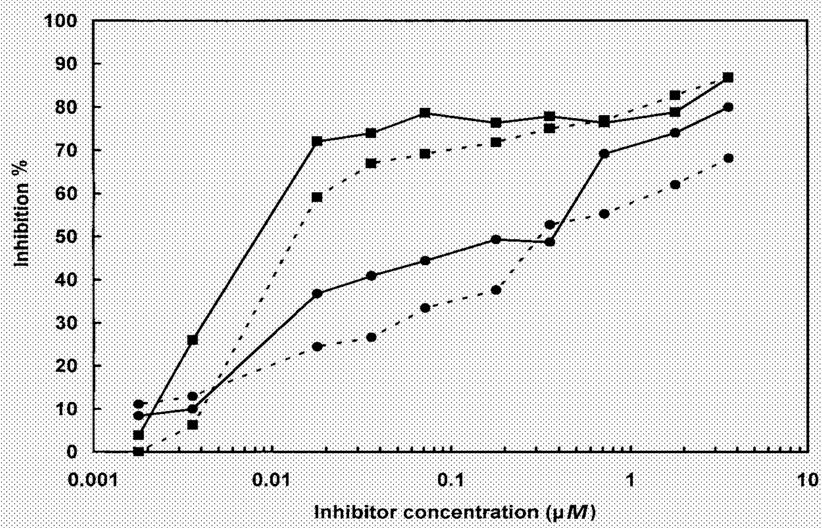

Fig. 9. Inhibition of IgE binding to natural Der p 1 or recProDer p 1. IgE binding to immobilized natural Der p 1 (dashed lines) or recProDer p 1 (solid lines) was inhibited by preincubation of a pool of serum (n = 20) with increasing concentrations of natural Der p 1 (■) or recProDer p 1 (●). Results are shown as 100% – (IgE binding in the presence of inhibitor/IgE binding in the absence of inhibitor).

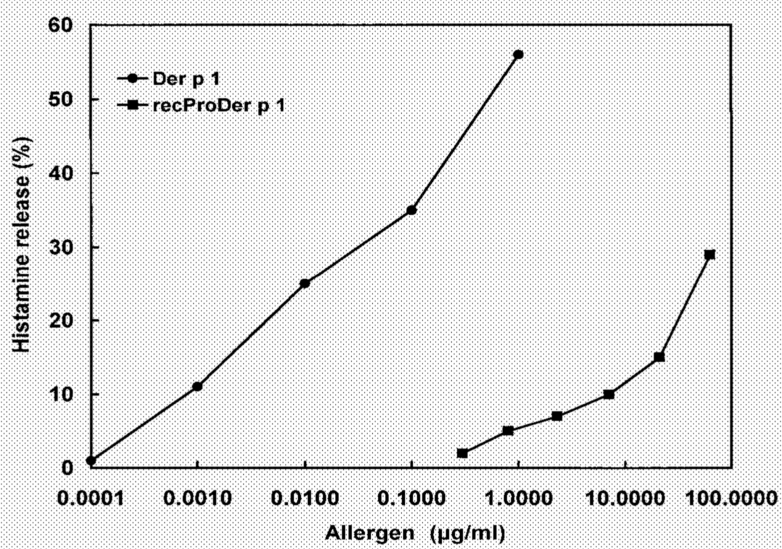

Fig. 10. Histamine release activity of recProDer p 1. Basophils isolated from the peripheral blood of one allergic donor were stimulated with serial dilutions of natural Der p 1 or recProDer p 1. The histamine released from cells was measured by ELISA. The total amount of histamine in basophils was quantified after cell disruption with the detergent IGEPAL CA-630. Results are shown as the ratio of histamine released by allergens to total histamine.

Indeed, natural Der p 1 was able to inhibit IgE binding to coated Der p 1 or recProDer p 1 more efficiently than the recombinant allergen used at the same concentrations. Whatever the type of coated allergen, a 50% inhibition of IgE binding was observed with a Der p 1 concentration of about 0.01 μ*M*. On the other hand, the same level of inhibition required a 30-fold higher recProDer p 1 concentration.

Histamine-Releasing Activity of recProDer p 1

To compare the allergenic activity of natural Der p 1 and recProDer p 1, basophils from one allergic patient were challenged in vitro with various concentrations of both allergens, and the released histamine was measured.

Natural Der p 1 was able to induce histamine release from basophils even at a concentration of 1 ng/ml. By contrast, recProDer p 1 could only release histamine at a

1,000-fold higher concentration (fig. 10). From this result, recProDer p 1 was shown to be less allergenic than the natural Der p 1.

Discussion

The inability to obtain large amounts of Der p 1, the major allergen from *D. pteronyssinus*, is a major obstacle for the development of biochemical and immunological studies. Indeed, whole mite culture is cost effective, the growth rate is slow and the purification yield of native Der p 1 is relatively low, about 1 mg of Der p 1 being purified from 1 g of whole mite culture in our experimental conditions. Moreover, previous attempts to express Der p 1 in bacteria and yeast indicated that the allergen was poorly expressed and mainly in an insoluble form [10–12]. In a recent paper, we successfully produced a recombinant precursor form of Der p 1, recProDer p 1, in *Drosophila* cells [13]. Our results suggested that, as with all cysteine proteinases, the Der p 1 proregion is probably essential for allergen processing.

The present study clearly reports that the production of recProDer p 1 in mammalian cells is very low, indicating that the presence of the prosequence is not sufficient to induce high-level recProDer p 1 expression.

Protein expression in mammalian cells is dependent on various parameters, such as the copy number of the transferred gene, transcriptional efficiency, mRNA processing and turnover, translation strength and protein stability [21]. It has been recently observed that expression efficiency can also be correlated with optimization of codon usage [22]. Conversion of low-usage codons to codons most frequently used in highly expressed human genes was shown to greatly increase the production level of several poorly expressed proteins in mammalian cells such as HIV-1 gag, env or bovine papillomavirus capsid proteins [23–25]. The enhancement of the level of expression was shown to be dependent on the availability of isoaccepting tRNAs [24, 26]. The codon prevalence of the ProDer p 1 gene was different from that most frequently used in highly expressed human genes. Two main approaches could be employed to optimize protein expression on the basis of the codon usage: synthetic genes could be produced with appropriate codon usage according to the expression systems used, or cells employed for expression could be modified at the level of rare tRNAs. A recent report demonstrated that modified *E. coli* cells with extra copies of certain tRNA genes expressed high levels of some peanut allergens affected by rare codon usage [27]. To assess the importance of appropriate codon usage for recProDer p 1 expression in CHO cells, we decided to engineer a synthetic ProDer p 1 gene based on the prevalent mammalian codons. Our results clearly demonstrate that codon optimization is beneficial to induce high-level expression of recProDer p 1 in mammalian cells. Divergence of preferential codon usage in *E. coli* and *Saccharomyces cerevisiae* with the pattern of ProDer p 1 codons could explain the low expression level of Der p 1 in bacteria and yeast. However, other factors could also influence the Der p 1 expression level, as recProDer p 1 was successfully expressed in insect cells, although codon usage in *Drosophila* is quite different from that of the ProDer p 1 gene.

Four chromatographic steps were combined to purify recProDer p 1 to homogeneity and N-terminal amino acid sequencing of purified recProDer p 1 products confirmed that maturation to Der p 1 did not proceed in CHO cells. The same situation was previously reported with recProDer p 1 expressed in *Drosophila* cells [13]. Carbohydrate analysis, performed with a set of specific lectins, showed that, contrary to Der p 1, recProDer p 1 was glycosylated. As expected, recProDer p 1 produced in mammalian cells carried more carbohydrate structures than that expressed in insect cells, a portion of recombinant molecules being fully glycosylated with terminal sialic acid residues.

recProDer p 1 immune recognition with anti-Der p 1 monoclonal conformational antibodies showed that recProDer p 1 displays very similar IgG binding characteristics compared with Der p 1. These results also confirmed that recProDer p 1 maturation is not a prerequisite for antibody binding.

The IgE reactivity of recProDer p 1 was investigated by direct ELISA, IgE inhibition and histamine-releasing activity assays. recProDer p 1 and natural Der p 1 displayed comparable IgE reactivities in direct assays for which both allergens were directly coated onto a solid phase. Similar results were also obtained when Der p 1 and recProDer p 1 were immobilized on plates with the help of an anti-Der p 1 monoclonal antibody [28]. These experiments could imply that the overall IgE-binding epitopes of natural Der p 1 are conserved in recProDer p 1 and that the presence of glycosylation and/or the prosequence in recProDer p 1 could have only a limited influence on its IgE reactivity. However, these results must be qualified by the data from competition ELISA and histamine release tests, which clearly indicated that recProDer p 1 displayed much lower IgE reactivity than does the natural Der p 1. Similar divergences in IgE reactivity were pre-

viously reported for native and recombinant Fel d 1 allergens [29]. A significantly reduced histamine-releasing capacity was also observed with a recombinant form of ProDer f 1 [30]. Possible explanations for the difference in IgE inhibition as well as histamine release between Der p 1 and recProDer p 1 could be: (1) one or several important IgE-binding epitopes of natural Der p 1 could be missing or denatured in recProDer p 1; moreover, one or several Der p 1 IgE-binding epitopes could be destructured or inaccessible when the natural allergen is directly coated on solid phases or immobilized with monoclonal antibodies, explaining the similar IgE-binding reactivity of both allergens when coated on solid phases, or (2) the specific IgE could display higher affinities for natural Der p 1 than for recProDer p 1. A recent paper demonstrated that the affinity of IgE antibodies to Der p 2 influenced allergen-induced histamine release [31]. A more detailed analysis to compare the IgE reactivity and the relative affinities of Der p 1 and recProDer p 1 is under way.

In summary, codon usage optimization can induce high-level expression of recProDer p 1, an allergen difficult to produce in CHO cells. This strategy could also be applicable for the expression of other allergens and could be extrapolated to other expression systems. Synthetic genes with appropriate codons could thus provide new tools for allergy diagnosis and specific immunotherapy.

recProDer p 1, immobilized on solid phases, could substitute natural Der p 1 in diagnostic tests for the detection of specific IgE. Considering the reduced recProDer p 1 anaphylactogenic potential, this recombinant allergen could be used in the future as an alternative reagent for immunotherapy to replace the commonly used allergen extracts. The immunogenicity of adjuvanted recProDer p 1 is currently under study in a murine model.

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